Quantitative decrease of human cytochrome *c* oxidase during development: evidences for a post-transcriptional regulation

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Abstract

In an earlier study, we showed that cytochrome *c* oxidase activity, measured in mitochondria isolated from human muscular biopsies, decreased steadily and substantially between the age of four years and adulthood ($P < 0.05$), whereas complexes I and III activity remained constant. The present study investigates a number of possible causes for this change in activity: although there is a drop in the apparent V_{max}, neither the apparent enzyme K_m, nor the cellular mtDNA concentration shows any variations over the studied period. Steady-state concentrations of mitochondrial gene transcripts (CO I, CO II, CO III, but also 12S, cytochrome *b*, or ND4) increase within this age group, indicating an overall increase in mitochondrial genome expression. Concentrations of transcripts of nuclear genes CO IV, CO Vb, and CO VIaH likewise show an increase, albeit less marked. On the other hand, heme aa3 levels and concentrations of mitochondrial (CO II) or nuclear (CO IV, CO VIIaH) subunits, estimated using specific antibodies, correlate closely with enzymatic activity and show a parallel decrease between 4 and 20 years. The observed decrease in complex IV activity is thus quantitative, and subject to post-transcriptional and/or post-translational regulation.

Keywords: Cytochrome *c* oxidase; Muscle; Mitochondrion; (Human)

1. Introduction

Cytochrome *c* oxidase or complex IV (ferrocytochrome-*c*:oxygen oxidoreductase, EC 1.9.3.1) is the last enzymatic complex of the mitochondrial respiratory chain. It catalyzes the transfer of four cytochrome *c* electrons to molecular oxygen, via a redox reaction.

In mammals, complex IV is made up of 13 different polypeptides [1,2]. Three subunits (CO I, CO II and CO III) are coded by the mitochondrial genome, the other ten being of nuclear origin. In man, two nuclear subunits, VIa and VIIa, present as two isoforms (L and H) [3–8]. These isoforms show tissue specificity, and their profiles of expression vary during development [9], in the course of myogenic differentiation [10].

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Nuclear subunits are involved in the regulation of complex IV enzymatic activity. Some of these subunits may modulate enzymatic activity via allosteric effectors (qualitative regulation) [11]. Moreover, certain nuclear subunits may have a limiting effect on assembly of the complex, thereby allowing modulation of its biosynthesis (quantitative regulation) [12,13]. This long-term regulation, which may occur at the transcriptional or translational level, enables enzyme quantities to be adapted as a function of given tissular requirements [14].

In an earlier study [15], we showed a very substantial decrease in complex IV activity in muscle mitochondria of children aged between 4 years and adulthood. This decrease occurs gradually, and is neither muscle- nor sex-dependent. Moreover, the decrease is strictly limited to complex IV, since enzymatic activities of citrate synthetase and of complexes I and III show no variations for this same age group. We thus sought to determine the possible causes of this variation in complex IV enzymatic activity during development. The research presented hereafter allowed us to ascertain that the variation in activity is mainly quantitative, and occurs during biosynthesis of complex IV.

2. Materials and methods

2.1. Origin of the biopsies

Muscular biopsies were taken during orthopedic surgery in the Pediatric Surgery Department of Hôtel-Dieu, Clermont-Ferrand (in keeping with the Authorization of the Consultative Committee for the Protection of Persons Undergoing Biomedical Research, Auvergne region). All fresh muscle specimens were those of a previous study where none of the studied muscles presented any anomaly in terms of structure or fibre distribution, and none of the children from whom specimens were taken presented disorders imputable to mitochondria. The reasons for surgery were most often injury or congenital malformations. Muscular biopsies were taken from six different muscles, and yield (milligram of protein from the mitochondrial fraction per gram of muscle), and enzymatic activities of citrate synthase, complexes I, III and IV, showed no significant difference in terms of muscle, in terms of sex, or in terms of age (except for complex IV). Age of the studied controls ranged between 3.5 and 22 years (mean = 11.2), with homogeneous distribution of the various ages [15].

Just after removal, a part of the biopsy (about 50–100 μg) was frozen in liquid nitrogen, and stored at -70°C until analyzed. The rest of the fresh tissue was used immediately, the mitochondrial isolation and the measurement of enzymatic activities (citrate synthase and cytochrome *c* oxidase) were performed in keeping with the procedure already described [15].

2.2. Kinetics studies

The rate of oxidation of horse ferrocytochrome *c* (Sigma) was measured at 550 nm with a Beckman DU8 spectrophotometer in KH_2PO_4 30 mM, EDTA 1 mM, pH = 7.4. Reduced cytochrome *c* was added to a final concentration of 5–50 μM. K_m (in μM) and V_{max} (in nmol/min · mg of mitochondrial proteins) values were determined from the straight-line Lineweaver–Burk plots by computer analysis of the data.

2.3. Extraction of nucleic acids

Total (cytoplasmic and mitochondrial) DNA and RNA were prepared from frozen tissues. Muscles were grounded in a lysis buffer (urea 7 M, Tris 10 mM, EDTA 10 mM, NaCl 0.3 M, pH = 8) and incubated 2 h at 56°C. After extraction of proteins with phenol/chloroform, DNA was recovered by ethanol precipitation and incubated 30 min with RNase at 37°C (RNase A). In a parallel manner, RNA was extracted from muscle with RNA-ZOL (Bioprobe System). Proteins were removed by phenol/chloroform extraction and RNA was recovered by ethanol precipitation. DNA and RNA concentrations in tissues extracts were estimated spectrophotometrically from A260.

2.4. Nucleic acid hybridization procedures

Total DNA was blotted directly to nylon filters (slot-blot), and total RNA were transferred by blotting from agarose formaldehyde gels. Nucleic acids

immobilized on nylon filters were hybridized to several probes labeled with [α-^{32}P]dCTP by random priming. Seven probes were obtained by PCR amplification of total muscle DNA with specific primers (Eurogentec Belgium): 12 S (sens 5′-GCCTAGCCACACCCCCACGGG-3′; anti-sens 5′-AGGAGGGTGACGGGCGG TGT-3′), CO I (sens 5′-CTACTAACAGACCGCAACCT-3′; anti-sens 5′-ACATAGTGGAAGTGGGCTAC-3′), CO II (sens 5′-CAAGTAGGTCTACAAGACGC-3′; anti-sens 5′-CCACAGATTTCAGAGCATTG-3′), ATP6-CO III (sens 5′-GTTCGCTTCATTCATTGCCC-3′; anti-sens 5′-GGATTATCCCGTATCGAAGG-3′), ND 4 (sens 5′-TACACTCACAACACCCTAGG-3′; anti-sens 5′-GGTGTATGAACATGAGGGTG-3′), cytochrome *b* (sens 5′-CCAATGACCCCAATACGCAA-3′; anti-sens 5′-AATGGGAGGTGATTCCTAGG-3′) and CO VIIaH (sens 5′-TGATCCGCTCCTTCAGCTCC-3′; anti-sens 5′-TTGTTCAAGTCTCTCAGGCCC-3′). All PCR products were purified (Geneclean, BIO 101), then used directly for random priming. For the ATP6-CO III amplification, the PCR product was first cut with Apa I, and the 190 bp fragment, specific to CO III, was isolated then purified before random priming.

The probes for nuclear messengers of complex IV (CO IV, CO Vb, CO VIaL, CO VIaH and CO VIIaL) are a gift from G. Bonne [9].

2.5. Immunoblotting

Quantitation of the three subunits (CO II, CO IV and CO VIIaH) in the mitochondrial fraction was performed by immunoblotting. Equivalent amounts of isolated mitochondria were incubated at 40°C for 2 h in a denaturing buffer (Tris-HCl 50 mM, pH = 6.8 containing 12% v/v glycerol, 1% p/v SDS, 4% v/v β-mercaptoethanol and 0.01% p/v Bromophenol blue) and loaded on a 12% polyacrylamide-SDS gel (18% for the CO VIIaH subunit). After migration, the proteins were electroblotted onto nitrocellulose membranes (0.1 μm pore size, Schleicher and Schull). Immunodetection of complex IV subunits was performed by incubation with primary antibodies (anti CO II is a gift from A. Lombes, anti CO IV is obtain from Molecular Probes and anti COP VIIaH is a gift from S. Possekel) at a dilution of 1:1000. Blots were analysed using goat anti-mouse IgG or goat anti-rabbit IgG conjugated to alkaline phosphatase at a dilution of 1:5000 (Sigma), followed by BCIP/NBT staining (Promega).

2.6. Hybridizations and immunoblotting signals analysis

Autoradiographies and stained membranes were scanned (OneScanner, Apple) and numeric images were analysed with a specific software (Graphteck, France) to quantify the intensity of each signal.

2.7. Heme aa3 concentration measurements

From the freshly isolated mitochondria, 100 μg of proteins are incubated 5 min at room temperature in 1% Triton X-100 (Sigma). Insoluble materials are removed by a brief centrifugation (5 min, 12 000 $\times$ g). The heme aa3 concentration (in pmol/mg) was calculated from difference spectra (dithionite-reduced minus air oxidized) with a Shimadzu UV 160-A spectrophotometer and using an extinction coefficient at 605–630 nm of 24 mM^{-1} cm^{-1}. The heme aa3 concentration was then expressed in citrate synthase units (ratio: heme aa3/citrate synthase activity) in order to obtain the amount of assembled enzyme per mitochondrial volume.

2.8. Statistical analysis

Statistical analysis were done as already described [15]. For comparison of data groups, the correlation was calculated to obtain the equation for the regression curves, the value of the correlation coefficient, and the statistical significance of correlation in keeping with the Fischer and Yates table. Results were validated as non-significant if $P > 0.05$, significant if $0.01 < P < 0.05$, very significant if $0.001 < P < 0.01$, and highly significant if $P < 0.001$.

3. Results

In a previous study, we showed that the age-related decrease in complex IV enzymatic activity is a phenomenon which occurs gradually during development, between the age of 4 years and adulthood [15]. We formulated several hypotheses and carried out

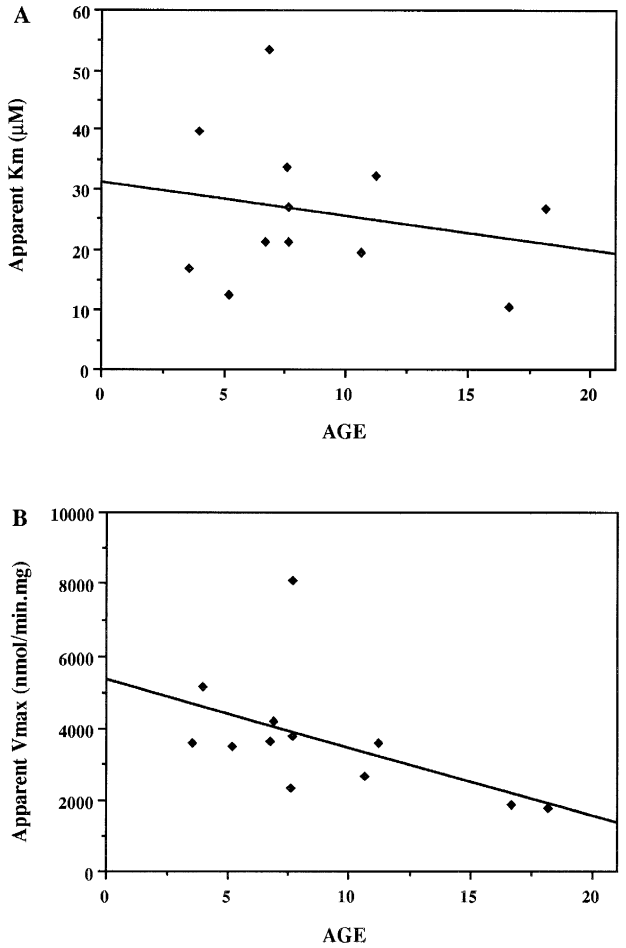

Fig. 1. Evolutions with age of kinetic parameters. Apparent K_m and apparent V_{max} were determined as described in Section 2. Statistical analysis were done showing a non-significant correlation ($P > 0.05$) for the K_m (A), and a highly significant correlation ($P < 0.01$) for the V_{max} (B). Lines represent regression curves.

several studies to determine the causes of this decrease. The variation in activity may be qualitative, i.e., a change in composition of the complex or of its environment, giving rise to a variation in enzymatic activity. The variation in activity may also be quantitative, in which case a decrease in the enzyme quantity (per citrate synthetase unit, thus per mitochondrium) would account for lowered activity.

3.1. Kinetic approach to complex IV

An analysis of correlation was performed between the calculated V_{max} and K_m values and age of subjects. The clusters of points and regression curves are shown on Fig. 1. Statistical analysis shows that the correlation is not significant for apparent K_m. On the other hand, the apparent V_{max} shows a highly significant correlation with age ($P < 0.01$). In adult biopsies, characterized by a lower complex IV activity, the enzyme quantity may be smaller than in biopsies from young children, characterized by high complex IV activity. This would account for V_{max} variations, despite the apparent lack of change in K_m.

3.2. Measurement of cytochrome levels

Measurements of the quantity of aa3 hemes by difference spectra were performed on the mitochondrial fraction of several biopsies. Because of the required protein quantities, only 12 biopsies could be analyzed. Values are expressed as citrate synthetase units (amount of assembled enzyme per mitochondrial volume), and the cluster of points and regression curve are shown on Fig. 2. As with the kinetic studies, the correlation between the concentration of aa3 hemes and age was analyzed, and proved to be statistically significant ($P < 0.05$). Young children (4 years), who show most enzymatic activity, present larger quantities of aa3 hemes than young adults (20 years), in whom enzymatic activity is not as high.

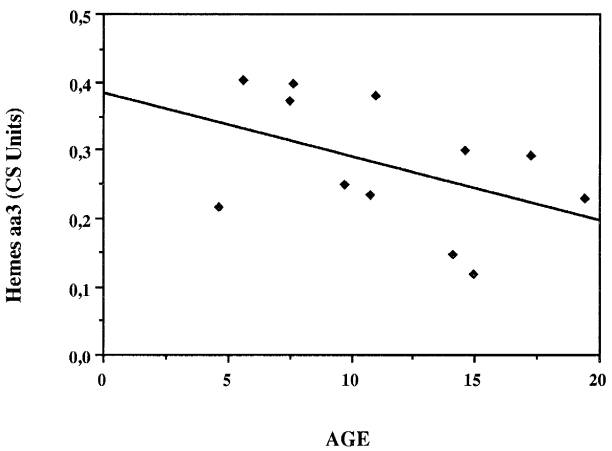

Fig. 2. Evolutions with age of hemes aa3 content. Cytochrome content measurements were done on isolated mitochondria and are expressed in citrate synthase units as described in Section 2. Statistical analysis were done showing a significant correlation ($P < 0.05$) and line represents regression curve.

The observed variations in complex IV activity with age are thus linked to quantitative variations in the cytochrome concentration.

3.3. Measurement of mtDNA levels

To study possible variations in mitochondrial gene concentrations, mtDNA levels of several biopsies were assessed using the 'Slot-blot' technique. The mtDNA/nuclear DNA signal ratio allows the quantity of mtDNA per nuclear genome to be assessed. The mtDNA/nuclear DNA ratio obtained for one biopsy (from the youngest child) was arbitrarily supposed to be equal to 1, and used as a reference for comparison with all the other biopsies.

A study was conducted to determine the correlation between mtDNA/nuclear DNA and subject age. The cluster of points and regression curve are shown on Fig. 3. The correlation is not statistically significant ($P = 0.48$); cellular levels of mtDNA can thus be considered as constant between 4 and 22 years.

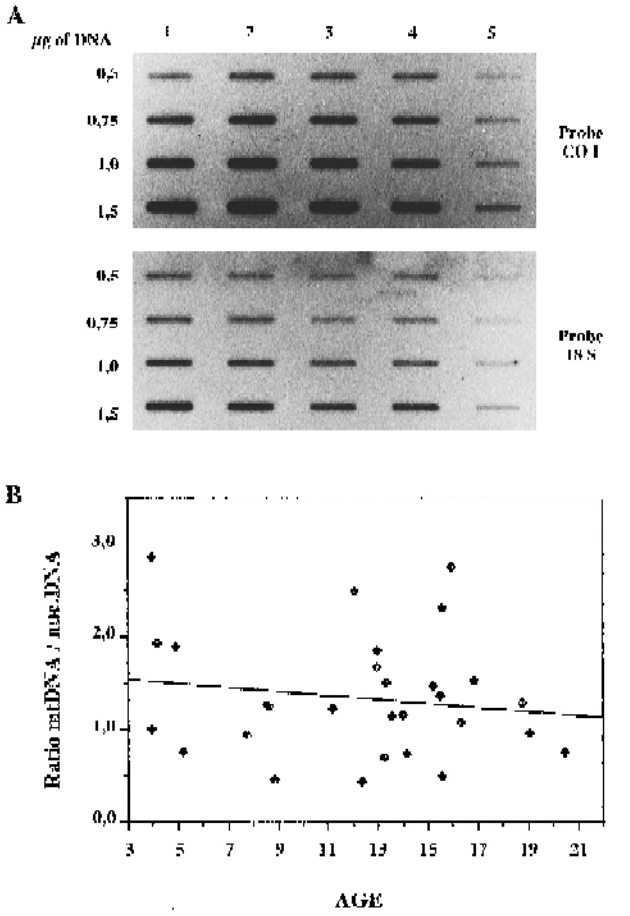

Fig. 3. Evolution with age of mtDNA/nuc. DNA ratios. Cellular mtDNA contents were estimated by slot-blot (A) from total DNA after RNase treatment. Correlation between mtDNA/nuc. DNA and age was statistically studied (B), showing a non-significant correlation ($P = 0.48$). Line represents regression curve.

3.4. Measurement of expression of several mitochondrial genes and complex IV nuclear genes

Levels of expression of several complex IV mitochondrial and nuclear genes as a function of age were assessed by Northern blot measurements based on total RNA extracts from 15 biopsies. To correct individual variations and allow comparison of the various hybridizations, measurements were performed for three different ages: 5 years, 10 years and 18 years (5 extracts for each age group). Hybridization signals obtained with the various probes were compared with the signal obtained using the 18S probe (Fig. 4A). Results are expressed as a function of the value obtained in 5-year-old children.

3.4.1. Mitochondrial gene expression

Evolution of steady-state concentrations of the three mitochondrial transcripts of complex IV (CO I, CO II, and CO III) as a function of age is shown on Fig. 4B. The relative steady-state concentration of these three transcripts shows an increase, with about 2.5 times more RNA at 18 years than at 5 years.

Expression of 12 S, cytochrome *b* and ND 4 genes as a function of age is shown on Fig. 4C. Just as with RNA from complexes IV, these levels show an increase, which is more marked for transcripts of complex I (ND 4) and III (cytochrome *b*) than for the ribosomal transcript (12S). The steady-state RNA concentration is about 3 times higher at 18 years than at 5 years, and is increased by 1.6 for 12 S.

Results thus confirm an overall increase in mitochondrial genome expression with age, whereas mtDNA levels remain constant. Nevertheless, we cannot speculate whether this increased expression is due to an increase in transcription or in messenger stability.

3.4.2. Expression of complex IV nuclear genes

Concentrations of nuclear transcripts of subunits CO IV, CO Vb, and CO VIaH were studied as a function of age. Results are presented on Fig. 4D.

There is no important variation in transcript concentrations, despite a slight increase in CO IV and CO Vb messenger levels. There is no clear evolution in CO VIaH, although this result may be imputable to quantification difficulties for this particular transcript. The variation is thus less marked for these transcripts than for mitochondrial RNA.

We studied expression of the L and H isoforms of nuclear subunits CO VIa and CO VIIa. For RNA of these subunits, we sought the presence of L isoforms in muscle extracts of children of different ages. Under our study conditions, given the limited availabil-

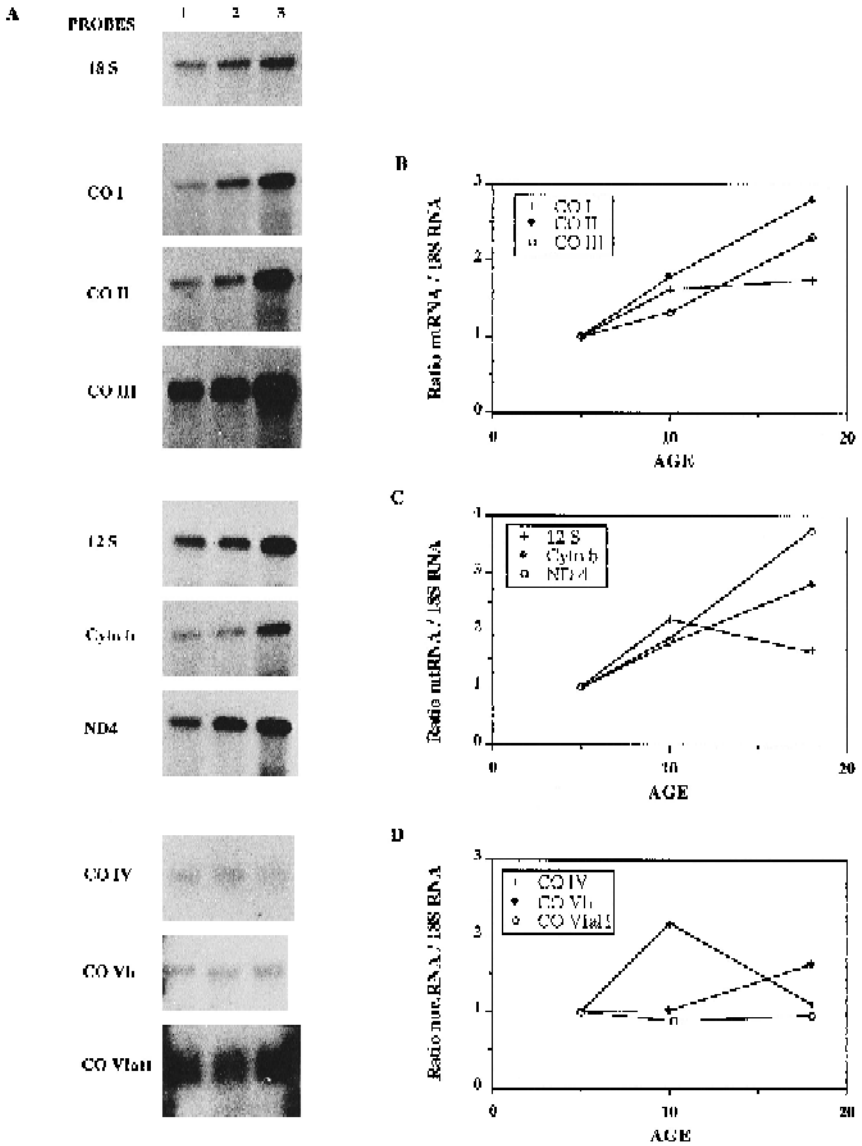

Fig. 4. Relative expression of several mRNA during development. Steady-state levels of ten RNAs were studied by Northern blot at three different ages (5, 10 and 18 years). Left panel (A), shows one of five independent experiments (lane 1: 5-year-old child, lane 2: 10-year-old child, lane 3: 18-year-old child, 10 μg of total RNA were used in each case). Hybridization signals obtained for the nine mRNAs were first divided by the signal obtained for the rRNA (18S), then expressed relatively to the ratio obtained in lane 1. Means were done within the five experiments and results are plotted in the right panel for the 6 mt RNAs (B and C) and the 3 nuclear RNAs (D).

ity of RNA quantities (10 μg per lane), these isoforms are undetectable, while H isoforms are present. There is thus apparently no important change in steady-state concentrations of VIaL and VIIaL RNA between 4 and 18 years, i.e., during the period for which we showed a decrease in complex IV activity. The disappearance of L isoforms in the muscle would thus seem to occur very early, before or during the neonatal period, or at the latest by the age of 4 years.

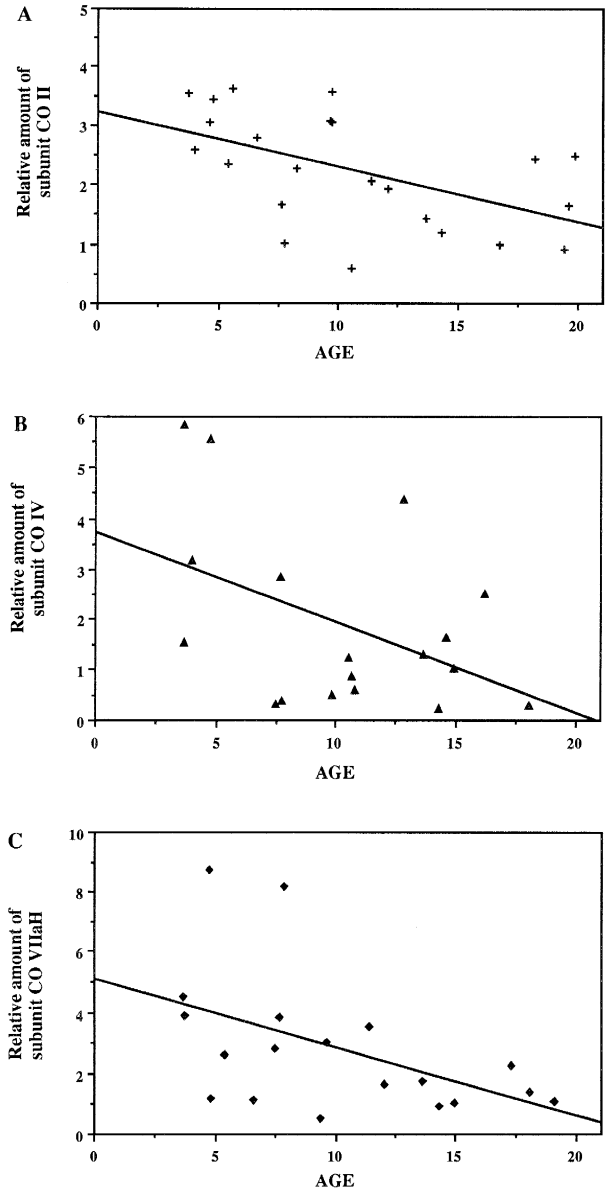

Fig. 5. Evolutions with age of complex IV subunits amounts. Relative amounts of complex IV subunit CO II (A), subunit CO IV (B) and subunit CO VIIaH (C) were estimated by Western blot using specific antibodies. In each case, one sample is chosen as reference, and others are expressed in relation to this reference. Statistical analysis were done showing a significant correlation in each case ($P < 0.05$) and lines represent regression curves.

3.5. Quantitative measurement of several complex IV protein subunits

A Western blot study of protein subunits CO II, CO IV and CO VIIaH of cytochrome *c* oxidase was carried out using specific antibodies. For these 3 subunits, a study was carried out to determine the correlation between relative staining intensity and age of subjects. The three correlation graphs and corresponding regression curves are shown on Fig. 5.

The correlation is statistically significant in the 3 cases, with $P < 0.01$ for subunit CO II and $P < 0.05$ for nuclear subunits CO IV and CO VIIaH. As with cytochrome levels, youngest children (4 years), characterized by the highest enzymatic activity, show the largest quantities of protein subunits. This result confirms the hypothesis of a quantitative decrease in complex IV between 4 years and adulthood.

Although the correlation between age and subunit quantities is more marked with the mitochondrial subunit (CO II) than with the two other subunits of nuclear origin, this difference is probably due to experimental conditions. Indeed, low molecular weights of the latter two subunits make them difficult to detect. Moreover, primary antibodies used for CO II are polyclonal, whereas those used for CO IV and CO VIIaH are monoclonal, reducing detection efficacy.

4. Discussion

The age-related decrease in complex IV activity has already been reported by several authors [16–23]. It has chiefly been described during senescence, and studies in the literature have rarely dealt with subjects younger than 20 years. In an earlier study, we showed a significant decrease in complex IV activity during human muscle development between the age of 4 years and adulthood. Research presented here allowed us to study several characteristics of complex

IV and of its biosynthesis, in order to determine the causes of this decrease.

4.1. Qualitative variation

The age-related decrease in enzymatic activity may reflect a variation in composition of the complex in terms of its subunits, which may lead to differences in catalytic performance. The three mitochondrial subunits (responsible for catalytic activity) are coded by the mitochondrial genome and present no isoforms. An intrinsic variation in complex IV should thus involve subunits of nuclear origin, and be dependent on age or stage of development. Recent research has shown such variations to exist in man, between the embryonic and adult stage, for two nuclear subunits, VIa and VIIa, which possess specific development isoforms [9,24]. Moreover, complex IV presents different kinetic parameters according to its L and H isoform composition [25].

Our study of transcripts isolated from biopsies shows that, as in adult muscle, there are practically no L isoform transcripts of VIa and VIIa genes, only H isoform transcripts being detectable. The adult form of muscular complex IV is already present at 4 years. The transition between L and H isoforms is therefore not responsible for the variation in activity observed after 4 years. Schillace and his collaborators [26] showed that transition occurs during the last fetal stages in bovines.

The variation in activity may also reflect modifications to kinetic parameters (V_{max} or K_m) of the complex, brought about by effectors which are extrinsic to the complex itself. Such effectors (intramitochondrial or cytosolic) may act on subunits of mitochondrial origin (catalytic) or of nuclear origin, such as ADP [27], ATP [28], or diiodotyronines [29]. The lipid environment of complex IV, notably membrane levels of cardiolipids, which may influence its enzymatic activity [30–32], could also be involved. Our kinetic parameter determinations indicate that the apparent K_m of complex IV remains constant between 4 and 20 years.

Although modulation of intrinsic activity by effectors (cytosolic and/or mitochondrial) cannot be ruled out altogether, decreased activity within this age group would not seem to be attributable to a major qualitative change in the actual complex.

4.2. Quantitative variation

Our results indicate that the decrease in enzymatic activity observed during development correlates with a decrease in the enzyme quantity, between 4 and 20 years. This is apparently not due to a reduction in the intramuscular mitochondrial population, since the extraction yield for isolated mitochondria showed no decrease; on the contrary, mitochondrial quantities per gram of muscle remained constant [15]. Moreover, citrate synthetase activity (per mg of protein) did not vary in the mitochondrial fraction isolated from children of different ages. Hence, the decrease in complex IV enzymatic activity indeed appears to result from a decrease in the intramitochondrial enzyme quantity.

This hypothesis is borne out by several results. Measurements of aa3 heme levels, and quantitative measurements of concentrations of several protein subunits (mitochondrial and nuclear) using specific antibodies show enzymatic activity to be directly related to the quantity of enzyme. The youngest children, who have the highest complex IV enzymatic activity, likewise show greater quantities of cytochromes and protein subunits than older control subjects, in whom enzymatic activity is lowest. The variation in activity between 4 and 20 years is therefore at least partially due to a quantitative decrease in the complex. This decrease in the intramitochondrial quantity of functional complex IV may be attributable to several factors, notably to regulation at one of the complex biosynthesis levels:

Analysis of mtDNA content shows that the variation in enzymatic activity is not attributable to a decrease in the quantity of information necessary for synthesis of the catalytic subunits. Van den Bogert et al. [33] tested various tissues and cell lines, and showed that there was no direct relation between mtDNA quantities and complex IV activity.

Our results obtained by studying various transcripts indicate an increase in the mitochondrial genome level of expression. This increase is shown not only for complex IV genes, but also for some genes of complexes I and III, which are not concerned by a decrease in enzymatic activities [15]. For these genes encoding complexes subunits, mRNA steady-state levels are about three times higher at 18 years than at 5 years, whereas the increase is only 1.6

fold for the rRNA 12S. For this ribosomal messenger, the steady-state level is 30 to 40 times higher than mRNA [34], which could explain the lower increase.

Expression of the studied complex IV nuclear genes likewise shows an increase, although it is less marked than for mitochondrial transcripts. For these two transcript populations, increased transcription or messenger stability, or a combination of both factors, may be involved. These results comply with several studies showing coordinated expression of the two genomes [9,33,35]. Furthermore, several authors have reported an increased level of transcription of all complex IV genes, between the human fetus and adult [9], and during development between birth and adulthood in the mouse [36].

The decrease in the quantity of complex IV therefore does not result from lowered expression of genes of this complex. Nevertheless, one or several nuclear messengers among those we have not studied may be present in limiting quantities, thereby regulating the quantity of synthesized complex IV.

There is no direct relation between steady-state transcript and protein levels. Identical results have already been published by various authors [37,38]. One or several mechanisms ensuring post-transcriptional or post-translational regulation must therefore be envisioned to account for the discrepancy between these two parameters [35].

The discrepancy may be attributable to a decrease in translation of mitochondrial mRNA of the complex, in which case the quantity of available, synthesized, catalytic subunits would constitute a limiting factor for functional complex IV assembly. In yeast, specific nuclear factors are involved in control of translation of several mitochondrial mRNA, notably CO III and cytochrome *b* [39,40]. No factor of this type is currently known in mammals. Several authors have nevertheless postulated the role of such factors, apparently operative at the level of interactions between mRNA and mitoribosomes [41,42], or at the level of protein synthesis initiation [43]; these factors might control the translation level of each of the mitochondrial messengers.

This phenomenon may alternatively be attributable to lowered nuclear mRNA translation (whereas steady-state concentrations show an increase), thus to a decrease in the quantity of nuclear subunits available in the mitochondria. Such a modulation in translation of these mRNA was proposed by Luis et al. [38]: these authors observed a possible accumulation of non-translated nuclear transcripts in the rat, during the antenatal period. On the other hand, they observed a sharp increase in β1ATPase protein synthesis in neonates, despite a stable transcript level. 'Relative translational efficacy' may thus vary as a function of development stage.

The discrepancy may also be due to a decrease in nuclear subunit imports into the mitochondria. Nevertheless, such a limiting stage would have to specifically affect subunits of this particular complex (there is no significant drop in complex I and III activity, [15]). Despite relatively comprehensive knowledge of protein import systems (see [44] for review), import specificity of this type has yet to be demonstrated.

Finally, the discrepancy may be attributable to lowered complex IV assembly capacity. Our method for estimating the quantity of complex IV subunits does not discriminate between those assembled within a functional complex and those present in the matrix or internal membrane. Although the role of chaperone proteins in the assembly process has been well elucidated (see [45] for review), the possibility of there being such specific regulation within the assembly has yet to be described.

Post-transcriptional or post-translational regulation probably plays an important role in the observed variation in cytochrome oxidase activity. This decrease in activity in the studied age group nevertheless raises the issue of correlative physiological manifestations: why is potential cytochrome oxidase activity in young subjects much higher than in older subjects. Is cytochrome oxidase activity involved in other processes in very young subjects (e.g., in heat regulation)? Does the decrease which has already been described after adulthood, namely during senescence, in fact occur earlier in development?

Better knowledge of factors possibly involved in coordinating and regulating the expression of nuclear and mitochondrial genes implicated in mitochondrial biogenesis should provide answers to these questions.

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